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Citation	Bioscience, biotechnology, and biochemistry (2014), 78(9): 1522-1528
Issue Date	2014-06-09
URL	http://hdl.handle.net/2433/198454
Right	The Version of Record of this manuscript has been published and is available inBioscience, Biotechnology, and Biochemistry (2014) http://www.tandfonline.com/10.1080/09168451.2014.923299.
Type	Journal Article
Textversion	author

Biosci. Biotechnol. Biochem.

Running title: 8-Anilinonaphthalene 1-Sulphonate and Thermolysin

Effects of Salts on the Interaction of 8-Anilinonaphthalene 1-Sulphonate and Thermolysin

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Abbreviations: AMPSO, 3-[(1,1-dimethyl-2-hydroxy-ethyl)amino]-2-hydroxypropane sulfonic acid; ANS, 8-anilinonaphthalene 1-sulphonate; DMSO, dimethyl sulfoxide; FAGLA, *N*-[3-(2-furyl)acrylol]-glycyl-L-Leucine; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOCAc-PLG, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly; MOCAc-PLGL(Dpa)AR, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu- [N^3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH_2

Neutral salts activate and stabilize thermolysin. In this study, to explore the mechanism, we analyzed the interaction of 8-anilinonaphthalene 1-sulphonate (ANS) and thermolysin by ANS fluorescence. At pH 7.5, the fluorescence of ANS increased and blue-shifted with increasing concentrations (0–2.0 μM) of thermolysin, indicating that the anilinonaphthalene group of ANS binds with thermolysin through hydrophobic interaction. ANS did not alter thermolysin activity. The dissociation constants (K_d) of ANS and thermolysin was 33 ± 2 μM at 0 M NaCl at pH 7.5, decreased with increasing NaCl concentrations, and reached 9 ± 3 μM at 4 M NaCl. The K_d values were not varied (31−34 μM) in a pH range of 5.5−8.5. This suggests that at high NaCl concentrations, Na^+ and/or Cl^- ions bind with thermolysin and affect the binding of ANS with thermolysin. Our results also suggest that the activation and stabilization of thermolysin by NaCl are partially brought about by the binding of Na^+ and/or Cl^- ions with thermolysin.

Key words: ANS; metalloproteinase; salt-induced activation; salt-induced stabilization; thermolysin

Thermolysin [EC 3.4.24.27] is a thermostable neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus*.[1,2] It consists of 316 amino acid residues with one zinc ion required for enzyme activity and four calcium ions required for structural stability.[3–6] X-ray crystallographic analysis has revealed that it consists of a β-rich N-terminal domain and an α-helical C-terminal domain.[7,8] It catalyzes specifically the hydrolysis of peptide bonds containing hydrophobic amino acid residues.[8,9] Thermolysin is widely used for peptide bond formation through reverse reaction of hydrolysis.[2]

Thermolysin activity increases with increasing concentrations of neutral salts in an exponential fashion.[10–16] The ratios of the specificity constant (k_{cat}/K_m) at 4 M NaCl to that at 0 M NaCl of thermolysin were 13–15 in the hydrolysis of neutral substrate *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide (FAGLA) and 6–7 in the hydrolysis of negatively charged substrate *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester (ZDFM) at pH 7.0 at 25°C.[10–16] Thermolysin stability increases with increasing NaCl concentration [NaCl] from zero to 1 M and then decreases with [NaCl] up to 4 M: the first-order rate constant, k_{obs}, of the thermal inactivation at 70°C, at pH 7.5, at 0, 1, and 4 M NaCl were 3.4, 1.2, and 2.8×10^{-4} s^{-1}, respectively.[12, 16] The ratio of the first-order rate constant (k_{obs}) of thermal inactivation at 70ºC at 0 M NaCl to that at 1 M NaCl of thermolysin was 3.[12] To explore the mechanism of salt-induced activation and stabilization of thermolysin, we made a preliminary X-ray crystallographic analysis of thermolysin in the presence of 4 M NaCl.[17] Two conformers in the active site were detected in the absence of NaCl, whereas only one was observed in the presence of 4 M NaCl. However, little is known about the mechanism.[17]

Several mutations that affect thermolysin activity and/or stability have been identified.[16,18–21] Of such mutations, Asp150→Glu markedly decreased the NaCl-induced activation and stabilization while Asn116→Asp did not exhibit any such marked decrease.[16] This suggests that the binding of Na^+ and/or Cl^- with certain residues of thermolysin is involved in the NaCl-induced activation and stabilization.[16]

8-Anilinonaphthalene 1-sulphonate (ANS) is a fluorescent probe.[22] It has hydrophobic and hydrophilic groups. It emits a large fluorescence energy when the anilinonaphthalene group binds with proteins through hydrophobic interaction, while it does not emit fluorescence when the sulphonic group binds with proteins through electrostatic interaction.[23] ANS is widely used for the analysis of proteins.[24–28] We previously reported the interaction of ANS and human matrix metalloproteinase 7 (MMP-7).[29]

In the present study, to explore the mechanism of salt-induced activation and stabilization of thermolysin, we analyzed the interaction of ANS and thermolysin. The results indicate that ANS binds with thermolysin through hydrophobic interaction and that the binding is affected by NaCl.

Materials and Methods

Materials. ANS (Lot CM10-118, 299.34 Da) was purchased from AnaSpec Inc. (San Jose, CA). The concentration of ANS was determined spectrophotometrically using the molar absorption coefficient at 350 nm, ε_{350}, of 5,000 M^{-1} cm^{-1}.[10,11] A three-times-crystallized and lyophilized preparation of thermolysin (Lot TIDC391, 34.6 kDa) was purchased from Daiwa Kasei (Osaka, Japan). The preparation was used without further purification. The thermolysin solution was filtered through a Millipore membrane filter, Type HA (pore size, 0.45 μm), before use. The concentration of thermolysin was determined using ε_{277} of 63,000 M^{-1} cm^{-1}.[10,11] FAGLA (Lot 111K1764) was purchased from Sigma (St. Louis, MO). The concentration of FAGLA was determined using ε_{345} of 766 M^{-1} cm^{-1}.[10,11] MOCAc-PLGL(Dpa)AR (1,093.2 Da)[30] and (MOCAc-PLG) (501.54 Da) were purchased from Peptide Institute (Osaka, Japan). Their concentrations were determined by the denoted molecular weights. All other chemicals were from Nacalai Tesque (Kyoto, Japan).

Fluorometric analysis of ANS. Pre-incubation (1,000 μl) was initiated by mixing 10 μl of the ANS solution (2,500 μM in 40 mM HEPES buffer at pH 7.5, 10 mM $CaCl_2$ (buffer A), 0–4.0 M NaCl, 0–490 μl of buffer A, and 0–500 μl of the thermolysin solution (4.0 μM in buffer A) at 25°C for 10 min. After the pre-incubation, the fluorescence spectra were measured with excitation at 380 nm and emission at 400-600 nm with a Shimadzu RF-5300PC fluorescence spectrophotometer at 25°C.

HPLC analysis of the thermolysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR. MOCAc-PLGL(Dpa)AR-hydrolyzing activity was determined by methods described previously.[31,32] Briefly, pre-incubation (240 μl) was initiated by mixing 0–50 μl of the ANS solution (2,500 μM in buffer A), 0–236 μl of buffer A, and 4 μl of the thermolysin solution (2.0 μM in buffer A) at 25°C for 10 min. The reaction was initiated by adding 10 μl of the substrate solution (0.5 mM) dissolved in DMSO to 240 μl of the pre-incubated solution (total volume 250 μl) at 25°C. The initial concentrations of thermolysin, substrate, ANS, and DMSO were 32 nM, 20 μM, 0−500 μM, and 4% v/v, respectively. The reaction was stopped at appropriate times, by mixing 100 μl of the reaction solution with 400 μl of 1% trifluoroacetic acid (TFA). This mixture (100 μl) was then applied to reversed-phase HPLC done on a TSKgel ODS-80Ts column (4.6 mm inner diameter x 150 mm) (Tosoh, Tokyo) equilibrated with 0.1% TFA, 20% v/v acetonitrile. A linear gradient was generated from 20 to 70% acetonitrile at a retention time of 5 min over 20 min at a flow-rate of 1.0 ml/min. The absorption of elutes was detected at 335 nm. The substrate and its two products, MOCAc-PLG and L(Dpa)AR, were separated, and they were evaluated by the respective peak areas. Reaction rate was determined from the time course of the production of MOCAc-PLG.

Thermal inactivation of thermolysin. Thermal inactivation of thermolysin was examined by methods described previously.[16] Briefly, 100 μL of a solution containing

1.0 μM thermolysin, in 50 mM acetate-NaOH buffer at pH 3.5, 4.0, and 4.5, 50 mM MES-NaOH buffer at pH 5.0, 5.5 and 6.5, 50 mM HEPES-NaOH buffer at pH 7.5 and 8.0 and 50 mM AMPSO-NaOH buffer at pH 8.5 and 9.0, each containing 10 mM $CaCl_2$ was incubated at 70ºC for specified durations (30, 60, 90, 120, 150, or 180 min). Then it was incubated at 25ºC for 5 min. Relative activity for FAGLA hydrolysis was determined as described above. Under the assumption that the thermal inactivation is irreversible and consists of only one step, the first-order rate constant (k_{obs}) of the inactivation was evaluated by plotting the logarithm of the activity (k_{cat}/K_m) against the duration of thermal treatment.

Spectrophotometric analysis of the thermolysin-catalyzed hydrolysis of FAGLA. FAGLA-hydrolyzing activity was determined by methods described previously.[10,11] Briefly, the reaction was initiated by adding 50 μl of the thermolysin solution to 950 μl of the substrate solution in buffer at various pH above described (total volume 1,000 μl) at 25°C. The initial concentrations of thermolysin and substrate were 0.1 μM and 400 μM, respectively. A_{345} of the reaction solution was measured continuously. The amount of FAGLA hydrolyzed was evaluated using the molar absorption difference due to hydrolysis, $\Delta\varepsilon_{345} = -310\ M^{-1}\ cm^{-1}$, at 25ºC.[10,11,20] Reaction rate was determined from the time course of the decrease of FAGLA.

Results

Effects of thermolysin on ANS fluorescence

Fluorescence spectra of ANS with varying concentrations of thermolysin in 40 mM HEPES at pH 7.5, 10 mM $CaCl_2$ (buffer A), 0 M NaCl were measured (Fig. 1). The shapes of the fluorescence spectra of ANS measured with 0−2.0 μM of thermolysin were almost the same, while the fluorescence intensity increased, and the wavelength giving the

Fig. 1

maximum fluorescence ($\lambda_{FI\max}$) decreased with increasing concentrations of thermolysin (Fig. 1(A)). The fluorescence intensity at 490 nm (FI_{490}) at 2.0 μM thermolysin was 133% of FI_{490} at 0 μM thermolysin (Fig. 1(B)). $\lambda_{FI\max}$ at 2.0 μM thermolysin was shorter by 14 nm than $\lambda_{FI\max}$ at 0 μM thermolysin (Fig. 1(C)). These results indicate that the anilinonaphthalene group of ANS binds with thermolysin through hydrophobic interaction.

Effects of ANS on thermolysin activity

FAGLA has been widely used as a substrate for thermolysin.[10–21,30] However, A_{345} detection was not available because of the effect of ANS. Accordingly, MOCAc-PLGL(Dpa)AR was used,[31] and the products were detected by reversed-phase HPLC (Fig. 2). Thermolysin and ANS were pre-incubated at 25°C for 10 min, followed by the reaction at 25°C. The reaction rates were unchanged with increasing concentration of ANS. This indicates that ANS neither activates nor inhibits thermolysin activity.

Fig. 2

Effects of salts on ANS fluorescence in the presence of thermolysin

Fluorescence spectra of ANS in the presence of 1.0 μM thermolysin in buffer A with varying concentrations of NaCl, NaBr, LiCl, or KCl were measured. The change of FI_{490}, ΔFI_{490}, defined as FI_{490} in the presence of 1.0 μM thermolysin minus FI_{490} in the absence of thermolysin, increased with increasing salt concentrations from zero to 4.0 M (Fig. 3(A)). ΔFI_{490} at 4.0 M NaCl, NaBr, LiCl, and KCl were 249, 204, 168, and 128%, respectively, of those at 0 M. In the presence of 1.0 μM thermolysin, $\lambda_{FI\max}$ at 4.0 M NaCl, NaBr, LiCl, and KCl were shorter by 17, 15, 11, and 8 nm, respectively, than $\lambda_{FI\max}$ at 0 M NaCl (Fig. 3(B)). In the absence of thermolysin, $\lambda_{FI\max}$ was unchanged (data not shown). Thus, $\Delta\lambda_{FI\max}$, defined as $\lambda_{FI\max}$ in the presence of 1.0 μM thermolysin minus $\lambda_{FI\max}$ in the absence of thermolysin, decreased from −6 to −17 nm with increasing NaCl concentration ([NaCl]), −6 to −15 nm with increasing [NaBr], −6 to −8 nm with increasing [LiCl], and

Fig. 3

−5 to −8 nm with increasing [KCl] from zero to 4.0 M. ΔFI_{490} and $\Delta\lambda_{FI\max}$ at 0−4 M NaCl are summarized in Table 1. These results indicate that the magnitude of the salt-induced changes in ANS fluorescence by 1.0 μM thermolysin at pH 7.5 was in the order of NaCl, NaBr, LiCl, and KCl, suggesting that Na^+ ion potently and Li^+ and K^+ ions slightly affect the ANS fluorescence in the presence of thermolysin, while Cl^- and Br^- ions hardly affect it.

Table 1

Binding of ANS with thermolysin at various NaCl concentrations

To measure the dissociation constants, K_d, of ANS with thermolysin, fluorescence area of varying concentrations of ANS was measured in the presence of 1.0 μM thermolysin at 0, 1.0, 2.0, 3.0, and 4.0 M NaCl, pH 7.5. The plot of [ANS]/Δ*FI*_area *vs.* [ANS] showed non-parallel lines intersecting near at the Y-axis (Fig. 4(A)). Under the assumption that ANS binds with thermolysin at a single site, the K_d values at pH 7.5 at 0, 1.0, 2.0, 3.0, and 4.0 M NaCl were calculated to be 33 ± 2, 29 ± 2, 23 ± 4, 15 ± 4, and 9 ± 3 μM, respectively. Relative K_d was defined as the ratio of the K_d value at x M NaCl to that at 0 M NaCl (33 ± 2 μM). The plot of the reciprocal of relative K_d *vs.* [NaCl] indicated that the reciprocal of relative K_d value increased with increasing [NaCl] (Fig. 4(B)). The increase in logarithmic value of the reciprocal of relative K_d value was not proportional to [NaCl] (Fig. 4(C)).

Fig. 4

Binding of ANS with thermolysin at various salts

To measure K_d of ANS with thermolysin, fluorescence area of varying concentrations of ANS was measured in the presence of 1.0 μM thermolysin at 4.0 M NaCl, NaBr, LiCl, or KCl at pH 7.5. The plot of [ANS]/Δ*FI*_area *vs.* [ANS] showed non-parallel lines which did not intersect (Fig. 5). The K_d values at pH 7.5 at 4.0 M NaCl, NaBr, LiCl, and KCl were calculated to be 9 ± 3, 10 ± 2, 12 ± 3, and 15 ± 2 μM, which were 27–45% of that of K_d without salts (33 ± 2 μM), indicating that the K_d values hardly depend on salt species at 4 M.

Fig. 5

Effects of pH on ANS fluorescence in the presence of thermolysin

Fluorescence spectra of ANS in the presence of 1.0 μM thermolysin at 0 M NaCl with varying pH were measured. ΔFI_{490}, defined as FI_{490} in the presence of 1.0 μM thermolysin minus FI_{490} in the absence of thermolysin, increased with increasing pH from 4.0 to 9.0, and ΔFI_{490} at pH 9.0 was 500% of that at pH 4.0 and 170% of that at pH 7.5 (Fig. 6(A)). In the presence of 1.0 μM thermolysin, $\lambda_{FI\max}$ at pH 9.0 was shorter by 17 nm than that at pH 4.0 and by 14 nm than that at pH 7.5, while in the absence of thermolysin, it was stable (Fig. 6(B)). Thus, $\Delta\lambda_{FI\max}$ decreased from −3 to −16 nm with increasing pH from 7.0 to 9.0. These results indicate that the magnitude of the change in ANS fluorescence by 1.0 μM thermolysin at 0 M NaCl increased with increasing pH from 7.0 to 9.0. Fig. 6

Binding of ANS with thermolysin at various pH

Thermolysin exhibits bell-shaped pH-activity profile with the maximum at pH 6.5 and 7.0.[20] Thermal stability of thermolysin at 70°C was examined at pH 4.5–8.5 (Fig. 7(A)). Relative stability was defined as the ratio of k_{obs} at pH 4.5 to that at given pH. The result indicated that the relative stability was highest at pH 6.5. Fig. 7

To measure K_d of ANS with thermolysin, fluorescence areas of varying concentrations of ANS were measured in the presence of 1.0 μM thermolysin at 0 M NaCl, pH 5.5, 6.5, 7.5, and 8.5. The plot of [ANS]/ΔFI_area *vs.* [ANS] showed non-parallel lines intersecting at the X-axis (Fig. 7(B)). Under the assumption that ANS binds with thermolysin at a single site, the K_d values at 0 M NaCl at pH 5.5, 6.5, 7.5, and 8.5 were 34 ± 3, 31 ± 2, 33 ± 2, and 32 ± 2 μM, respectively (Fig. 6(B)), indicating that the K_d values were unchanged with varying pH from 5.5 to 8.5.

Discussion

It has been observed that the effectiveness of monovalent cations of neutral salts to activate thermolysin is in the order of $Na^+ > K^+ > Li^+$, being different to that of the Hofmeister series ($Li^+ > Na^+ > K^+$).[10,33,34] Based on this evidence, the activation of thermolysin by neutral salts is not derived from changes in water structure or in the hydration of the enzyme or substrate. Hence it has been speculated that the salt-induced activation of thermolysin might be as a result of conformational changes brought about by the direct interactions of the ions with charged residues on the enzyme.[10–15]

In this study, we showed that ANS binds with thermolysin (Fig. 1), ANS does not inhibit thermolysin activity (Fig. 2), and NaCl and other salts increase the affinity of thermolysin for ANS (Figs. 3–5 and Table 1). The degree of the salt-induced changes in ANS fluorescence by thermolysin was in the order of NaCl, NaBr, LiCl, and KCl (Fig. 3 and Table 1), suggesting that Na^+ affects ANS fluorescence more potently than Cl^- ion. It also suggests that the effectiveness of monovalent cations of neutral salts on the salt-induced changes in ANS fluorescence by thermolysin is $Na^+ > Li^+ > K^+$, being different to that of activation of thermolysin ($Na^+ > K^+ > Li^+$). The K_d values of ANS and thermolysin at 4.0 M salt were both in the order of KCl, LiCl, NaBr, and NaCl (Fig. 5), suggesting that the degree of the effects of salts on the interaction of ANS and thermolysin depends on salt species. From the result that the K_d values of ANS and thermolysin did not change with increasing pH from 5.5−8.5 (Fig. 7), we speculate that amino acid residues with side chains with pK_a values of around 5.5−8.5 might not be located in the ANS-binding site. However, from the results presented in this study, it is difficult to precisely speculate the mechanism of the effects of salts on the interaction of ANS and thermolysin.

In contrast to that little is known about the mechanism of salt-induced activation of thermolysin, effects of ions on enzyme activity have been well studied in human immunodeficiency virus type-1 (HIV-1) protease. HIV-1 protease is a homodimeric aspartic protease, each containing 99 amino acid residues. Like thermolysin, HIV-1 is highly activated and stabilized by neutral salts (1－2 M NaCl).[35–37] Recent molecular

dynamics simulations and conductivity measurement analysis of HIV-1 protease have shown that Na^+ binds at least twice as strongly to the surface of HIV-1 protease than K^+ does.[38,39)]

In conclusion, this study suggests that Na^+ and/or Cl^- ions bind with thermolysin and affect its binding with ANS. We think that the activation and stabilization of thermolysin by neutral salts might be due to the binding of ions with thermolysin, and that ANS might be useful as a fluorescent probe for studying the interaction of ions to thermolysin. The binding sites for ANS were determined in some proteins.[40,41)] The elucidation of the binding site of thermolysin for ANS is the next research subject.

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Figure legends

Fig. 1. Effect of Thermolysin on the Fluorescence of ANS.

Fluorescence spectra were measured with excitation at 380 nm and emission at 400-600 nm for 25 μM ANS in buffer A with various concentrations of thermolysin at 25ºC. (A) Fluorescence spectra with 0–2.0 μM thermolysin. (B) FI_{490} with 0–2.0 μM thermolysin. (C) Wavelength giving the maximum fluorescence ($\lambda_{FI\max}$). Error bars indicate SD values of triplicate measurements.

Fig. 2. Effect of ANS on Thermolysin Activity.

Thermolysin was pre-incubated for 10 min in the presence and absence of ANS at 25°C at pH 7.5, and the reaction was carried out with 32 nM thermolysin and 20 μM MOCAc-PLGL(Dpa)AR at 25°C at pH 7.5. Error bars indicate SD values of triplicate measurements.

Fig. 3. Effect of Salts on the Fluorescence of ANS.

Fluorescence spectra were measured with excitation at 380 nm and emission at 400-600 nm for 25 μM ANS in buffer A, 0–4.0 M NaCl (hollow circle), NaBr (hollow triangle), LiCl (hollow square) or KCl (hollow diamond) in the presence and absence of 1.0 μM thermolysin at 25ºC. (A) Change in fluorescence intensity at 490 nm (ΔFI_{490}). ΔFI_{490} was defined as the difference in FI_{490} between the values in the presence and the absence of 1.0 μM thermolysin. ΔFI_{490} was plotted against salt concentration. (B) The wavelength giving the maximum fluorescence ($\lambda_{FI\max}$). $\lambda_{FI\max}$ in the presence of 1.0 μM thermolysin was plotted against salt concentration. Error bars indicate SD values of triplicate measurements.

Fig. 4. Effect of NaCl on the Binding of ANS with Thermolysin.

Δ *FI*_area was defined as the difference in FI_{490} between the values in the presence

and the absence of 1.0 μM thermolysin. Δ*FI*_area were measured with 10–250 μM ANS in the presence of 0 (solid circle) 1.0 (hollow diamond), 2.0 (hollow square), 3.0 (hollow circle), or 4.0 M (hollow circle) NaCl. [ANS]/Δ*FI*_area *vs.* [ANS] plot is shown. (B) Effect of NaCl on the dissociation constants (K_d). Relative K_d was defined as the ratio of the K_d value at x M NaCl to that at 0 M NaCl (30 ± 2 mM^{-1}). (C) Logarithmic relationship of K_d with [NaCl]. Error bars indicate SD values of triplicate measurements.

Fig. 5. Effect of Salts on the Binding of ANS with Thermolysin.

Δ*FI*_area were measured with 10–250 μM ANS in the presence of 4 M NaCl (hollow circle), NaBr (hollow triangle), LiCl (hollow square) or KCl (hollow diamond) and in the absence of salts (solid circle). [ANS]/Δ*FI*_area *vs.* [ANS] plot is shown. One of the representative data is shown.

Fig. 6. Effect of pH on the Fluorescence of ANS.

Fluorescence spectra were measured with excitation at 380 nm and emission at 400-600 nm for 25 μM ANS in 50 mM acetate-NaOH buffer at pH 3.5, 4.0, and 4.5, 50 mM MES-NaOH buffer at pH 5.0, 5.5 and 6.5, 50 mM HEPES-NaOH buffer at pH 7.5 and 8.0 and 50 mM AMPSO-NaOH buffer at pH 8.5 and 9.0, each containing 10 mM $CaCl_2$ at 25°C. (A) Change in fluorescence intensity at 490 nm (ΔFI_{490}). ΔFI_{490} was defined as the difference in FI_{490} between the values in the presence and the absence of 1.0 μM thermolysin. ΔFI_{490} was plotted against pH. (B) The wavelength giving the maximum fluorescence ($\lambda_{FI\max}$). $\lambda_{FI\max}$ in the presence (hollow square) and the absence (hollow circle) of 1.0 μM thermolysin was plotted against pH. Error bars indicate SD values of triplicate measurements.

Fig. 7. Effect pH on the Binding of ANS with Thermolysin.

(A) pH-stability profile. Thermolysin (1.0 μM) in 40 mM HEPES-NaOH, 10 mM

$CaCl_2$, and 0–4.0 M NaCl at pH 7.5 was incubated at 70°C for specified durations. The experimental conditions for FAGLA hydrolysis were as described in the Materials and methods section. Relative stability of thermolysin variants was defined as the ratio of the first-order rate constant, k_{obs}, of the thermal inactivation at 0 M NaCl ($1.2 \pm 0.1 \times 10^{-4}$ s^{-1}) to that at x M NaCl. (B) [ANS]/Δ*FI*_area *vs.* [ANS] plot. Symbols for pH: 5.5, hollow circle; 6.5, hollow triangle; 7.5, hollow square; and 8.5, solid circle. Error bars indicate SD values of triplicate measurements.

Table 1. Effect of Salts on Thermolysin Acitivity and Change in ANS Fluorescence by Thermolysin.

Salt		Relative activity[a]	ΔFI_{490}[b]	$\Delta\lambda_{FI\max}$[b]
no salt		1.0	2.2	–6
LiCl	1 M	1.1	2.9	–10
	2 M	1.5	3.0	–10
	3 M	3.4	3.2	–11
	4 M	4.5	3.6	–11
NaCl	1 M	1.9	3.0	–8
	2 M	3.6	3.7	–9
	3 M	6.9	5.0	–10
	4 M	13	5.9	–17
KCl	1 M	1.7	2.5	–6
	2 M	3.0	2.2	–7
	3 M	5.2	2.8	–11
	4 M	9.0	2.6	–8
NaBr	1 M	1.8	2.7	–11
	2 M	3.2	3.1	–12
	3 M	5.8	4.2	–15
	4 M	11	4.7	–15

[a] Ref. 15. The relative activities are the ratios of the specificity constant, k_{cat}/K_m, in the hydrolysis of FAGLA at x M NaCl to that at 0 M NaCl, in 40 mM Tris-HCl buffer at pH 7.5, 10 mM $CaCl_2$ at 25°C. The k_{cat}/K_m value at no salt is 2.2×10^4 M^{-1} s^{-1}.

[b] The ΔFI_{490} and $\Delta\lambda_{FI\max}$ were determined based on the results shown in Fig. 3.

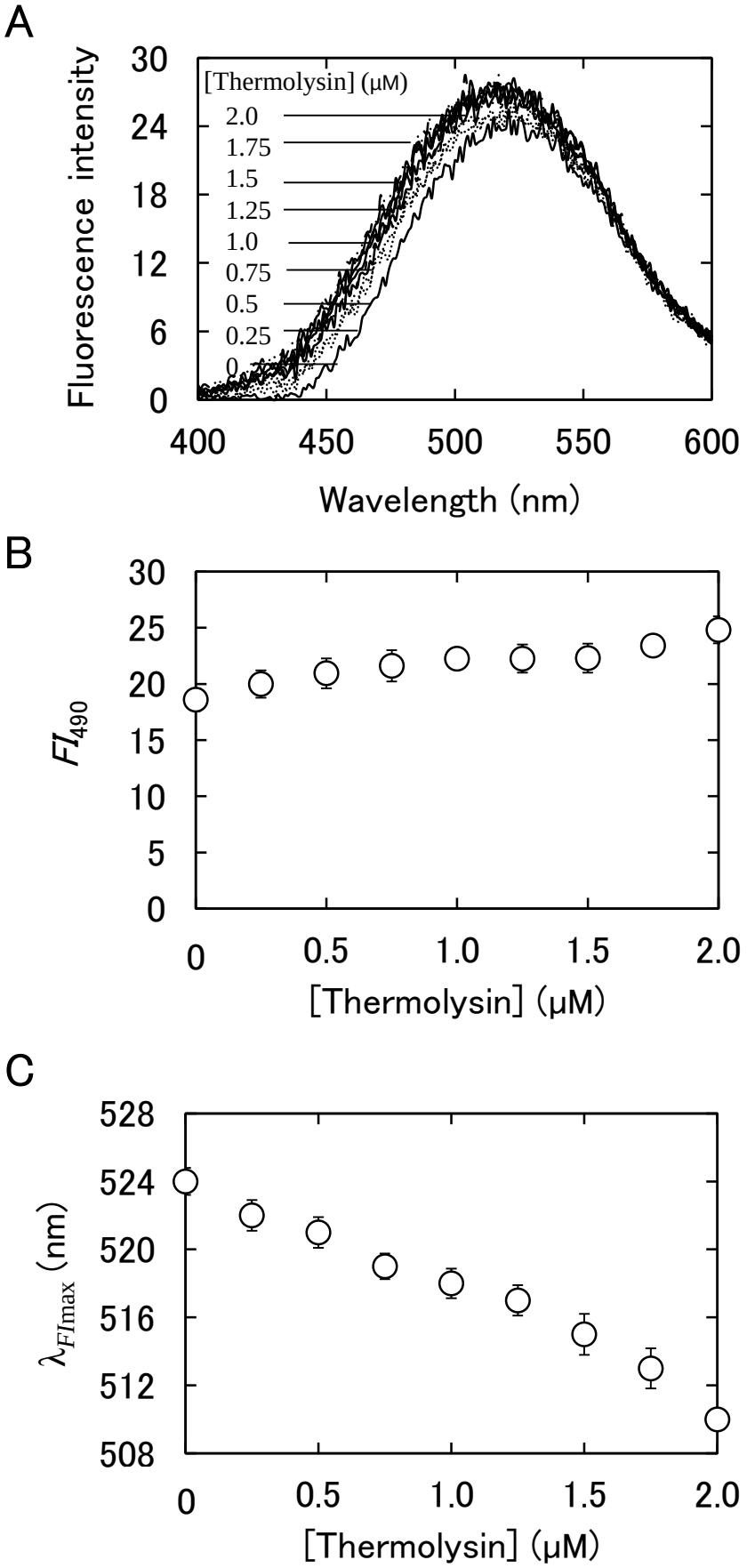


Fig. 1, Samukange *et al*.

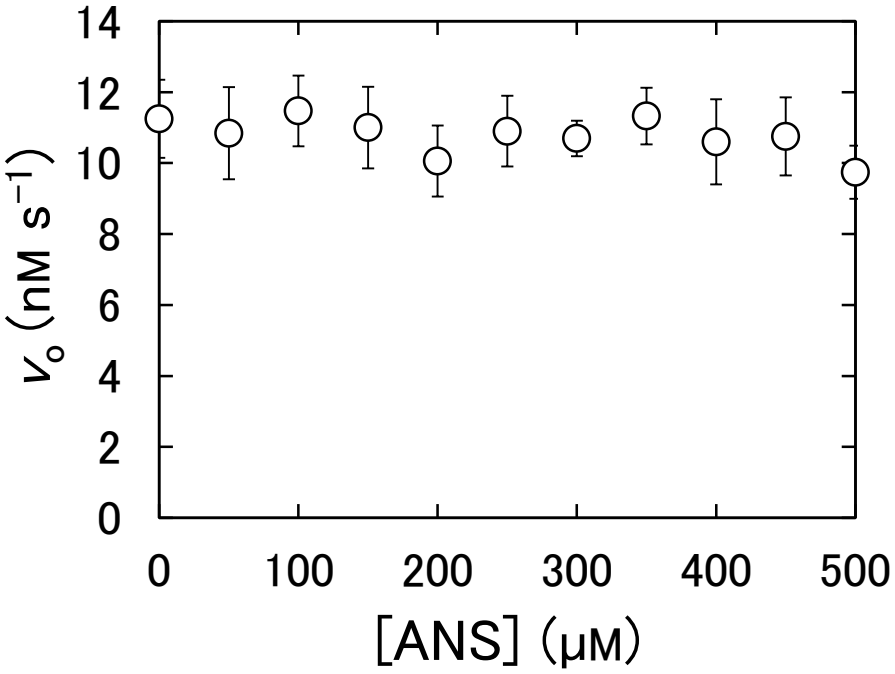


Fig. 2, Samukange *et al*.

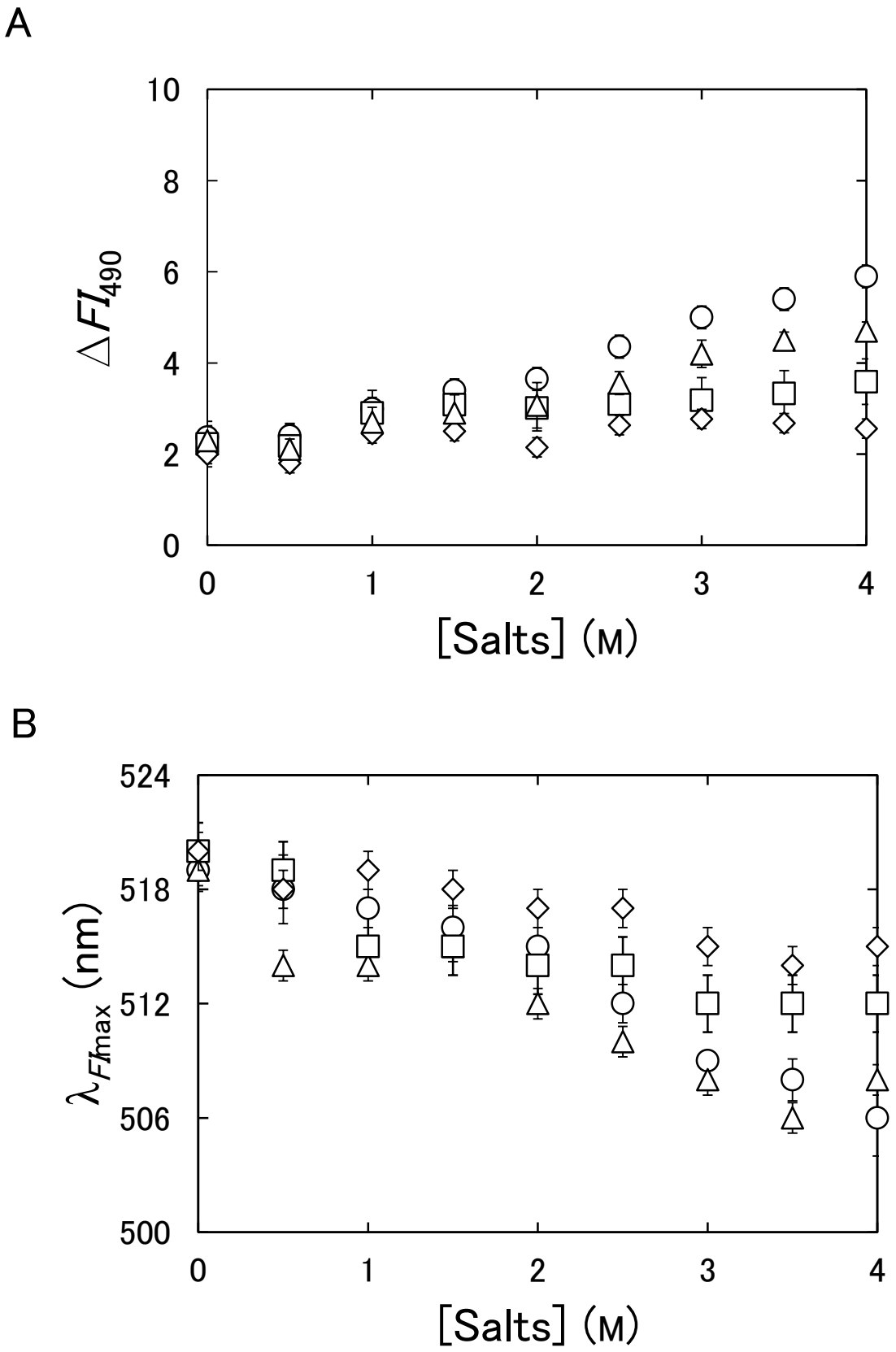


Fig. 3, Samukange *et al*.

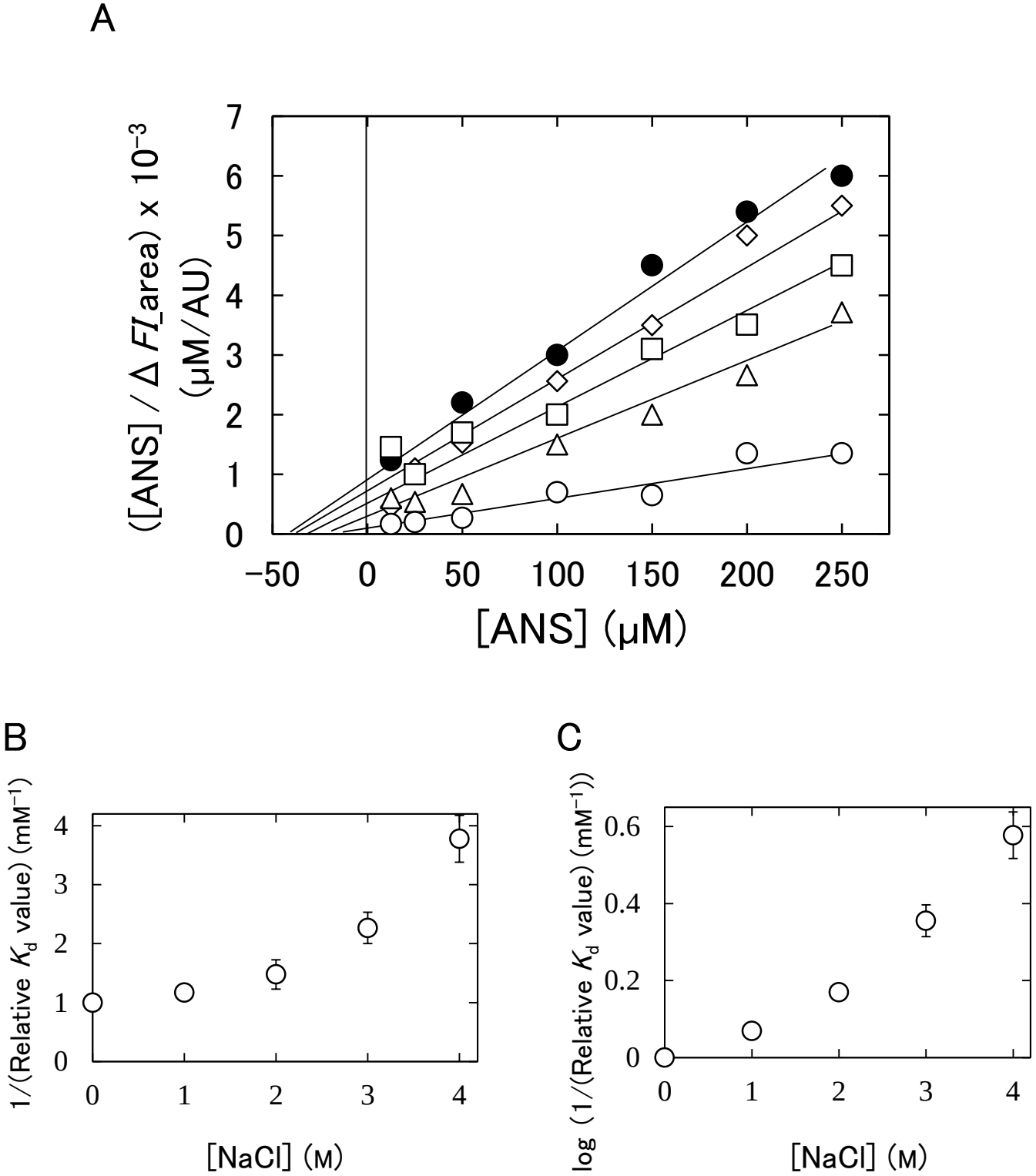


Fig. 4, Samukange *et al*.

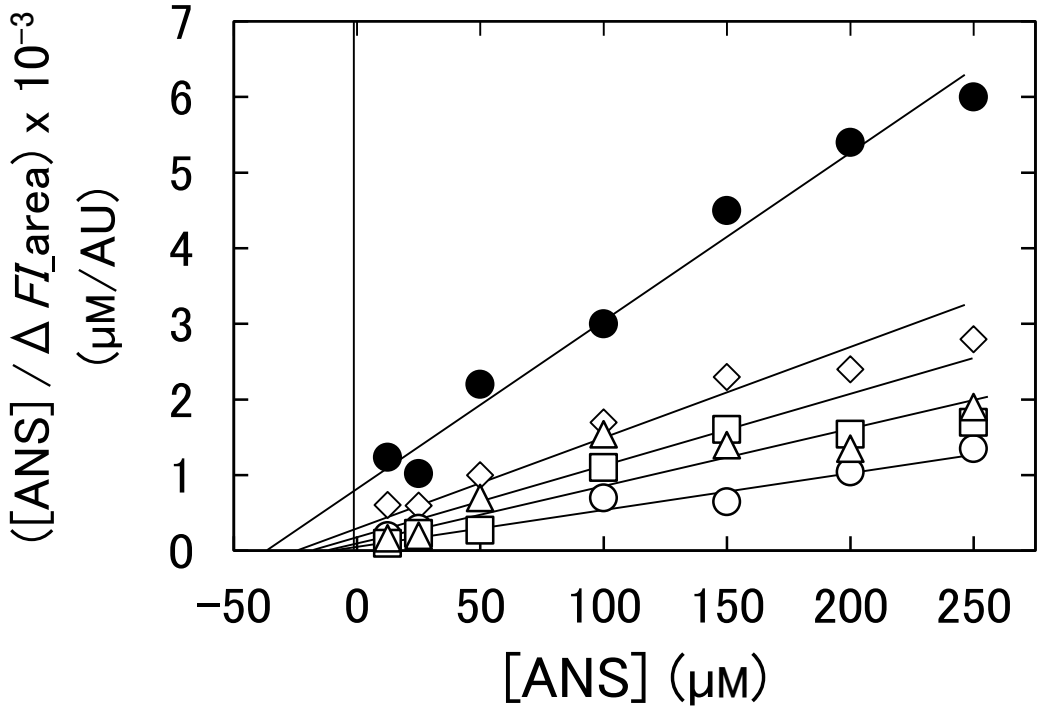


Fig. 5, Samukange *et al*.

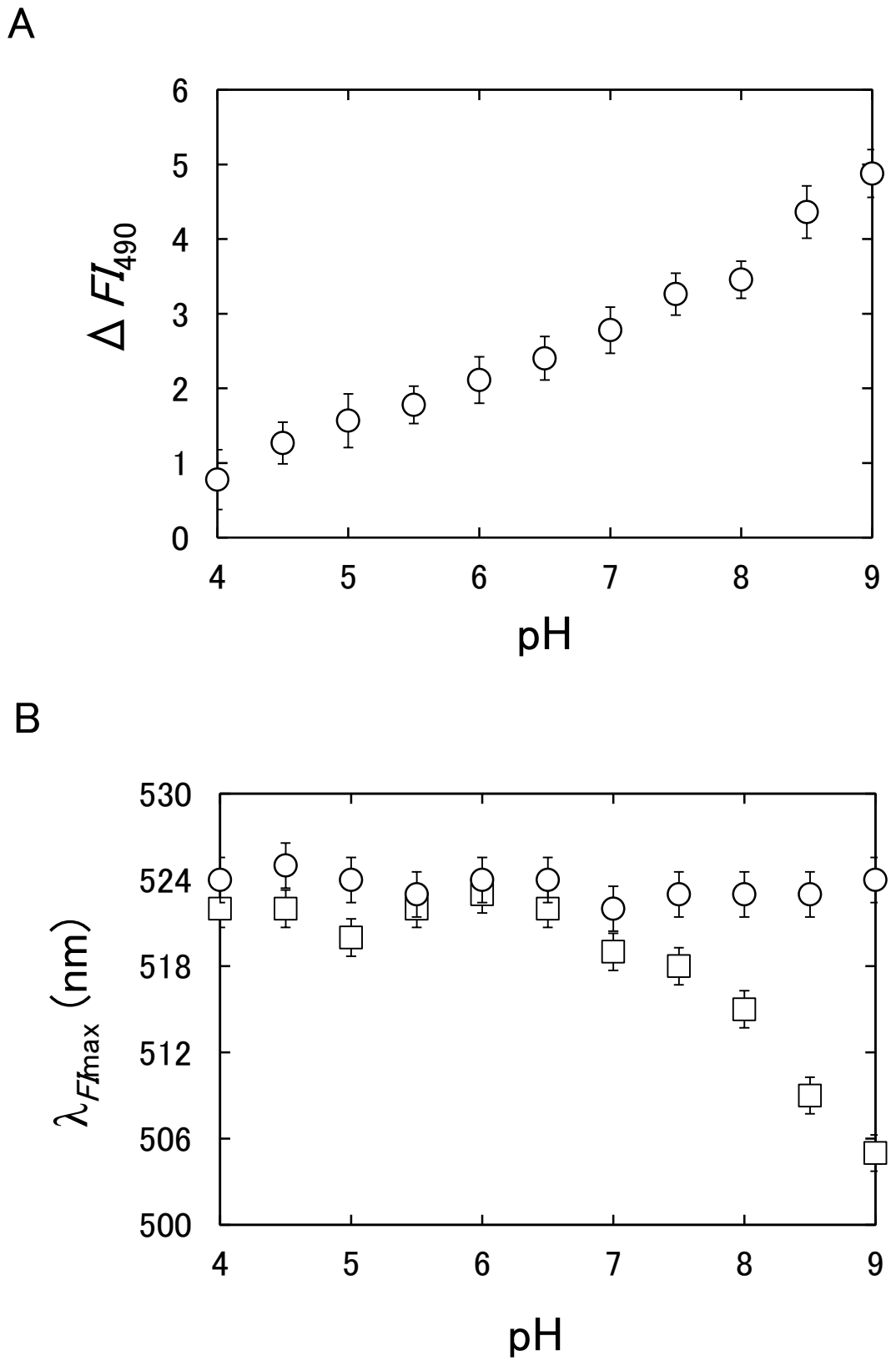
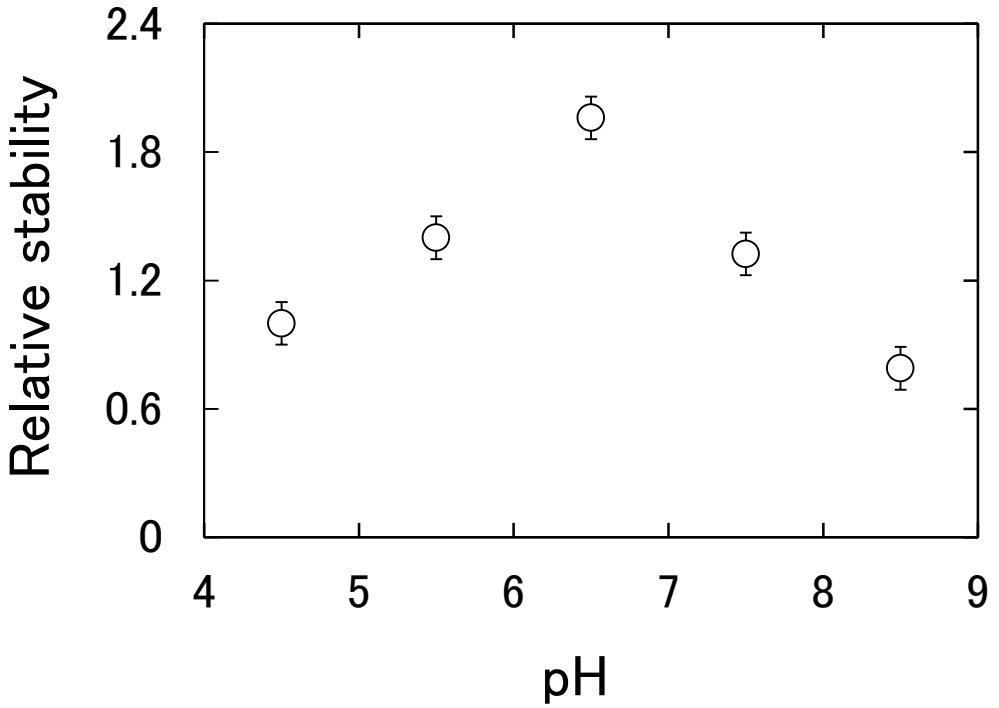


Fig. 6, Samukange *et al*.

A



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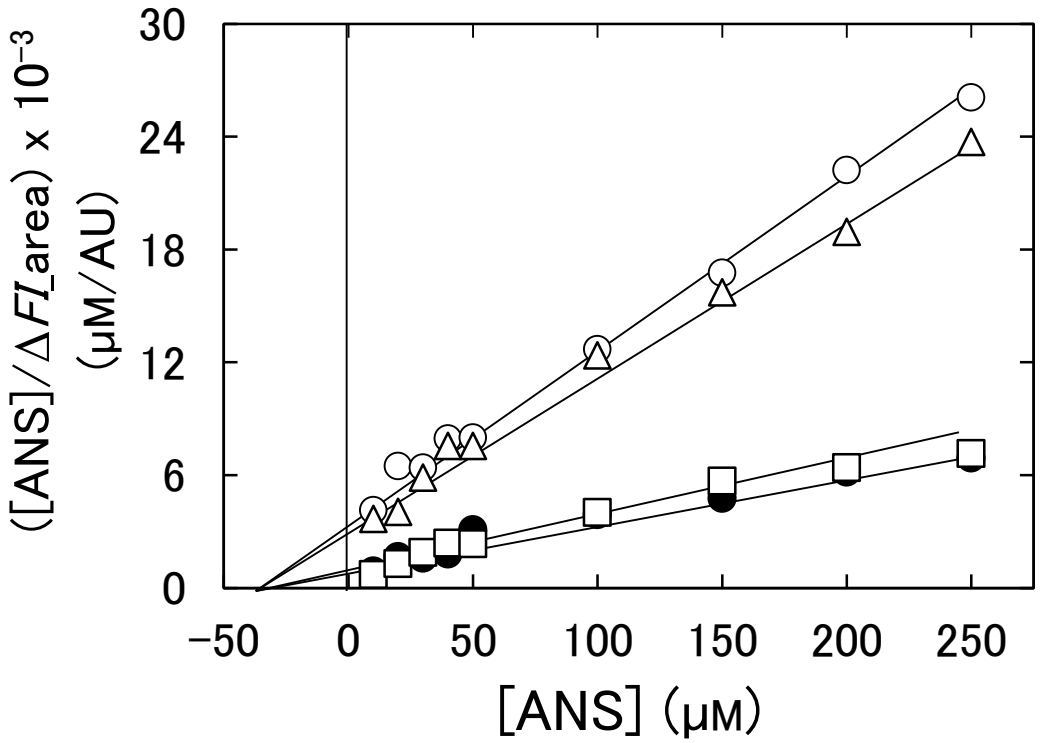

Fig. 7, Samukange *et al*.